The use of rubisco DNA sequences to examine the systematic position of *Hernandia albiflora* (C.T.White) Kubitzki (Hernandiaceae), and relationships among the Laurales

Effie M. Ablett,[1] Julia Playford[2] and Stephanie Mills[1]

Summary

Ablett,Effie M., Playford,Julia and Mills,Stephanie (1997). The use of rubisco DNA sequences to examine the systematic position of *Hernandia albiflora* (C.T.White) Kubitzki and relationships among the Laurales. *Austrobaileya* 4(4): 601–607. The DNA sequence of the large subunit of the chloroplast enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L), has been determined for *Hernandia albiflora* and *Doryphora aromatica* (F.M.Bailey) L.S.Sm. (Monimiaceae). Bootstrap analyses of these sequences and those from related species were carried out using parsimony, distance, and maximum likelihood methods. *Hernandia albiflora* is very strongly grouped with *Hernandia ovigera* L. (100%) in all three analyses. This supports its placement in the Hernandiaceae and not in the Lauraceae. The analyses also supported traditionally recognised phylogenies. For example, the Laurales are distinct from other magnoliid orders which are represented in our analyses by *Eupomatia* and *Amborella*. Within the Laurales Hernandiaceae, Lauraceae, Calycanthaceae and Monimiaceae are distinct clades. The molecular analyses differ from Cronquist (1988) in the inclusion of *Idiospermum* with the Calycanthaceae (supported by 99–100% of bootstrap replicates in our analyses), and the weak association of *Gyrocarpus* with the Hernandiaceae (supported in less than 65% of bootstrap replicates). Our analyses also suggest that *Doryphora* may be in a separate clade from *Hedycarya*, but this needs to be examined further by the inclusion of sequence data from more species of Monimiaceae.

Keywords: Calycanthaceae, Hernandiaceae, Lauraceae, Monimiaceae, *Doryphora, Hernandia albiflora, Hernandia ovigera, Eupomatia , Amborella, Gyrocarpus, Hedycarya.*

[1]*Centre for Molecular and Cellular Biology, University of Queensland (current address for EMA: Queensland Institute of Medical Research)*
[2]*Cooperative Research Centre for Tropical Rainforest Ecology and Management and Department of Botany, University of Queensland.*

Introduction

Recently, there has been a blossoming of DNA-based analyses in studies of plant systematics and evolutionary genetics. Because of its fundamental importance in photosynthesis, the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), has been studied extensively throughout the plant kingdom. The DNA sequence of the large subunit chloroplast gene (*rbc*L) has been used to infer phylogeny in higher plants (Palmer et al. 1988; Martin and Dowd 1991). Many laboratories have concentrated their efforts on coordinated *rbc*L sequencing studies (Olmstead et al. 1992; Duvall et al. 1993; Soltis et al. 1990; and Martin and Dowd 1991), resulting in a large-scale analysis of 500 seed plant species (Chase et al. 1993). The results of two separate analyses show good agreement with the morphological classification in the placement of taxa in familial groups.

Hernandia albiflora (C.T.White) Kubitzki is a rare plant of north Queensland rainforests. In the past there has been some doubt as to the classification of *Hernandia albiflora* based on morphological criteria. *Hernandia albiflora* was first described by White (1935) as *Valvanthera albiflora* (C.T.White). The new monotypic genus, *Valvanthera*, was tentatively placed in the Hernandiaceae, with some reservation, as some features suggested affinities with the Lauraceae. It has since been placed in the Lauraceae (Hutchinson 1964) and Hernandiaceae (Kubitzki 1969).

We have determined the sequence of the *rbc*L gene in *Hernandia albiflora* and *Doryphora aromatica* (F.M.Bailey) L.S.Sm. to provide information on the position of *Hernandia albiflora*. These sequences were

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analysed using cladistic methods to determine whether investigation of the evolutionary changes in DNA will result in a more precise definition of the phylogenetic position of *Hernandia albiflora* and to see if additional sequences will give more information on the relationships among the Laurales.

Materials and methods

The phylogenetic position of *Hernandia albiflora* and *Doryphora aromatica* in relation to the other Laurales was examined by analysing the DNA sequences we obtained along with those obtained by other workers (see **Table 1**). Other Magnoliid species (i.e. *Eupomatia bennettii* F.Muell and *Amborella trichopoda* Baill. sensu Chase et al. 1993) were included to see if the Laurales were a distinct group, and *Podocarpus gracilior* Pilg. was selected as a remote outgroup.

***Sources of DNA samples*:** DNA was extracted from leaves of *Hernandia albiflora* and *Doryphora aromatica,* which had been transported as voucher specimens for up to one week, and then frozen at -80°C. Additional leaves from the same samples were identified and stored as vouchers at the Queensland Herbarium (see Table 1 for AQ numbers). Total cellular DNA was purified using cetyl trimethylammonium bromide (CTAB) buffer, chloroform:isoamyl alcohol extraction, and alcohol precipitation (Doyle and Dickson 1987).

***DNA sequencing*:** The coding sequence of the *rbc*L gene was amplified using PCR and primers based on known conserved regions as described by Olmstead et al. (1992). In the case of *Doryphora aromatica,* these primers gave non-specific products, and the reverse primer 5' CTTCACAAGCAGCAGCTAGTTCAGGACTCC 3' (sequence supplied by M.Chase, personal comm.) was used to amplify 1325 bp of the coding sequence.

The PCR products were purified using 'Gene Clean' (Bresatec) or 'Magic PCR Preps' (Promega), and subjected to direct double stranded dideoxy sequencing using Zurawski *rbc*L sequencing primers (Zurawaski pers. com. DNAX Research Institute of Molecular and Cellular Biology). The reverse primers 153R, 346R, 674R, 1020R, and 1375R did not work well for these species and the new reverse primers shown in **Table 2** were used for either manual sequencing with 'Sequenase Version 2' kits (U.S. Biologicals) or automated sequencing with Applied Biosystems 'model 373A' and 'Taq Dye Deoxy Terminator Cycle Sequencing Kit'. The modified manual protocol involved heating the annealing mix to 100°C and cooling in ice (rather than NaOH denaturation), labelling at 0°C with increased concentrations of ^{35}S-labelled ATP and reduced concentrations of the other nucleotides; and extension at 45°C. For automated sequencing, increasing the PCR annealing temperature from 50°C to 58 °C or 60 °C was found to improve the sequence profile obtained using reverse primers from 686 to the 3' end.

All sequences were aligned by comparison to the sequence for *Hernandia ovigera* L. using 'AssemblyLIGN' (version 1.0.3, International Biotechnologies Inc.), and confirmed by reverse strand sequencing using different PCR preparations.

***Phylogenetic analysis*:** Three separate analyses were carried out using completely different methods to derive trees. Parsimony analyses were performed using PAUP version 3.1.1 (Swofford 1993). The branch and bound bootstrap search was used with the following settings: no upper bound, furthest data addition sequence, an initial maxtrees setting of 100, branches having maximum length zero were collapsed and no topological constraints were used. Maximum likelihood and distance analyses were carried out using PHYLIP (Felsenstein 1989) and the Australian National Genomic Information Service's Sun 4/670 computer. Bootstrap analyses (Felsenstein 1985) were carried out to place confidence levels on the inferred phylogenies. Bootstrap replicates of data were generated using 'seqboot', and subject to analysis by 'dnadist' and 'neighbour' using the default settings (with 'm' set at 100).

Results of phylogenetic analyses

The results of bootstrap analyses using parsimony (PAUP), distance (PHYLIP), and maximum likelihood (PHYLIP) are shown in Figures 1, 2 and 3 respectively.

Table 1. DNA sequences used in this study.

Species	Family	Voucher No.	RbcL sequence start-stop	Source
Amborella trichopoda	Amborellaceae	A	29-1428	A
Calycanthus chinensis	Calycanthaceae	A	29-1428	A
Calycanthus floridus	Calycanthaceae	A	27-1428	A
Chimonanthus praecox	Calycanthaceae	A	29-1428	A
Eupomatia bennettii	Eupomatiaceae	A	29-1428	A
Gyrocarpus sp.	Hernandiaceae	A	27-1428	A
Hernandia ovigera	Hernandiaceae	A	29-1428	A
Valvanthera albiflora	Hernandiaceae	AQ546699	27-1428	L77210*
Idiospermum australiense	Idiospermaceae	A	29-1428	A
Cinnamomum camphora	Lauraceae	A	29-1428	A
Persea americana	Lauraceae	A	1-1428	A
Doryphora aromatica	Monimiaceae	AQ546705	31-1370	L77211*
Hedycarya arborea	Monimiaceae	A	29-1428	A
Podocarpus gracilior	Podocarpaceae	A	1-1428	A

A = see Chase et al. 1993
* = Genebank sequence number
AQ numbers are shown for voucher specimens held at Queensland Herbarium (BRI).

Table 2. New *rbc*L sequencing primers. New primers based on the sequence of a more closely related species, *Hernandia ovigera*. (Genebank L12650).

235R 5' CTC GAT GTG GTA GCA TCG TCC TTT GTA ACG 3'

686R 5' TGT CCT TTG ATT TCA CCT GTT TCG GCC 3'

436R 5' ATG CCA TGA GGG CGG CCT TGG AAA GTT 3'

1131R 5' TAT GCC AAA CGT GAA TAC CCC CTG AAG 3'

All of the analyses gave similar cladograms. In each case *Amborella* and *Podocarpus* were excluded from the Laurales group in 98% or 100% of the bootstrap replicates. When *Eupomatia* was included (**Figs 1, 2**) it was only loosely associated with the Laurales, and this reflects the taxonomic classification of the Magnoliales and the Laurales in Magnoliidae.

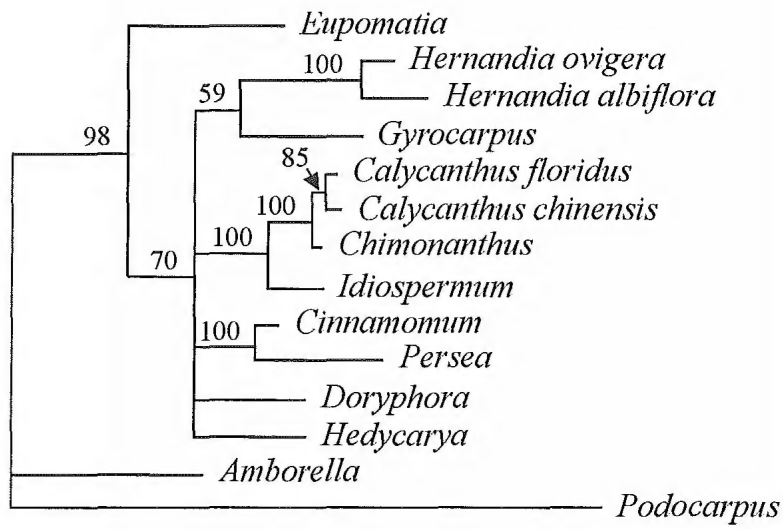

Fig. 1. Parsimony analysis of *rbc*L sequences. The 50% majority tree of a 100 replicate bootstrap analysis is shown. Confidence estimates of groups are indicated by the numbers (percentage of bootstrap replicates with that particular group). See Table 1 for species information.

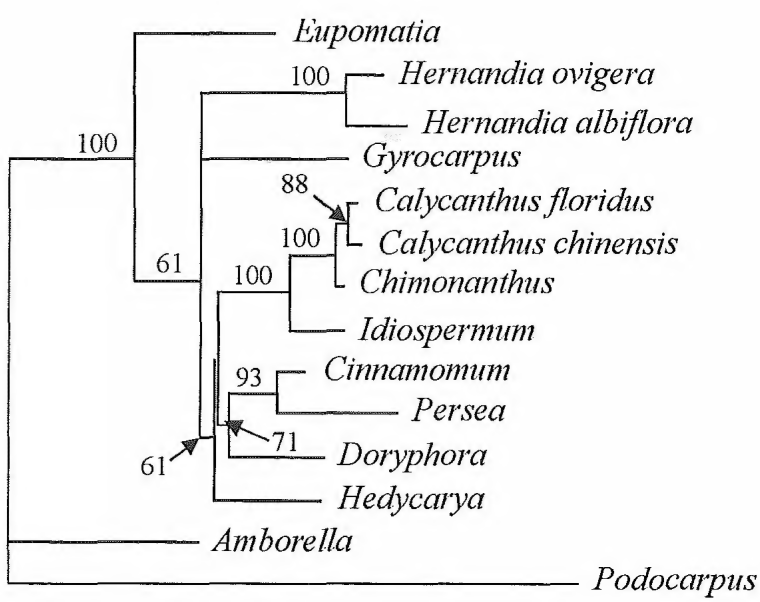

Fig. 2. Distance analysis of *rbc*L sequences. The 50% majority tree of a 100 replicate bootstrap analysis is shown. Confidence estimates of groups are indicated by the numbers (percentage of bootstrap replicates with that particular group). See Table 1 for species information.

Within the Laurales, similar associations resulted from all three analyses.

As in the analyses of Qui et al. (1993), the Laurales fall into four distinct clades which also reflect traditional classification: (i) *Hernandia/ Gyrocarpus*, (ii) Calycanthaceae (*Calycanthus floridus* L., *Calycanthus chinensis* Cheng. & S.T.Chang and *Chimonanthus praecox* (L.) Link/*Idiospermum australiense* (Diels) S.T.Blake), (iii) the Lauraceae (*Cinnamomum camphora* (L.) T.Nees & Eberm. and *Persea americana* Mill.) and (iv) *Hedycarya*. In our parsimony and maximum likelihood analyses, *Doryphora* appeared as a fifth clade distinct from the other Monimiaceae species, *Hedycarya*. The association of *Doryphora* with the Lauraceae in the distance analysis is only weakly supported (71%).

Hernandia albiflora is very strongly grouped with *Hernandia ovigera* (100%) in all three analyses. This strongly contradicts its placement in the Lauraceae.

Discussion and implications

Evolution is ultimately a process resulting from changes in the sequence of DNA in the genome. Until recently, genetic alterations could be inferred only from the phenotype. Now, with the advent of molecular taxonomy, changes in DNA sequence can be used to monitor evolutionary changes directly, rather than inferring changes from the resultant morphological end-points. Molecular systematics has the potential to infer phylogeny where there are gaps in the gradation of morphological features in living and fossil species.

Using this approach, we have analysed DNA sequence data from relic species to investigate relationships within the Laurales. All analyses presented here provide strong evidence (100% of bootstrap replicates) that *Hernandia albiflora* belongs in Hernandiaceae and not in Lauraceae. We have undertaken three different types of analysis (parsimony, distance, and maximum likelihood), and obtained concordant results, both for the placement of *Hernandia albiflora* and the groupings within the Laurales. These analyses are also concordant with those of Chase et al. (1993) and Qui et al. (1993), which reflect traditionally recognised phylogenies. For example, the Laurales are distinct from other Magnoliid orders (represented in our analyses by *Eupomatia* and *Amborella*). Within the Laurales the Hernandiaceae, Lauraceae, Calycanthaceae and Monimiaceae are distinct clades. Also, with a small set of species we were able to use much more rigorous analyses and present further verification of the large analyses of Chase et al. (1993) and Qui et al. (1993).

The molecular analyses digress from Cronquist (1988) in the inclusion of *Idiospermum* with the Calycanthaceae (supported by 99% to 100% of bootstrap replicates in our analyses), and the weak association of *Gyrocarpus* with the Hernandiaceae (supported in less than 65% of bootstrap replicates). This indicates that *Gyrocarpus* could be a sister group to the Hernandiaceae.

Our analyses also suggest that *Doryphora aromatica* may be in a separate clade from *Hedycarya arborea* J.R.Forst & G.Forst., and raise the possibility that the Atherospermataceae are more closely related to the Lauraceae than to the other Monimiaceae sub-groups. This needs to be examined further by the inclusion of sequence data from more species of Monimiaceae.

We have presented some preliminary findings that indicate analysis of rubisco DNA sequences can be used to define the systematic position of species such as *Hernandia albiflora* where the classification is in doubt. Rubisco sequencing can also be used to investigate relationships among relic species.

This approach should be particularly useful when dealing with primitive taxa extinction because of species makes it difficult to interpret evolutionary relationships using morphological features. Phylogenetic relationships may be better understood by molecular analysis of the remaining taxa.

It is important to sequence the remaining relic taxa in order to investigate their phylogenetic relationships.

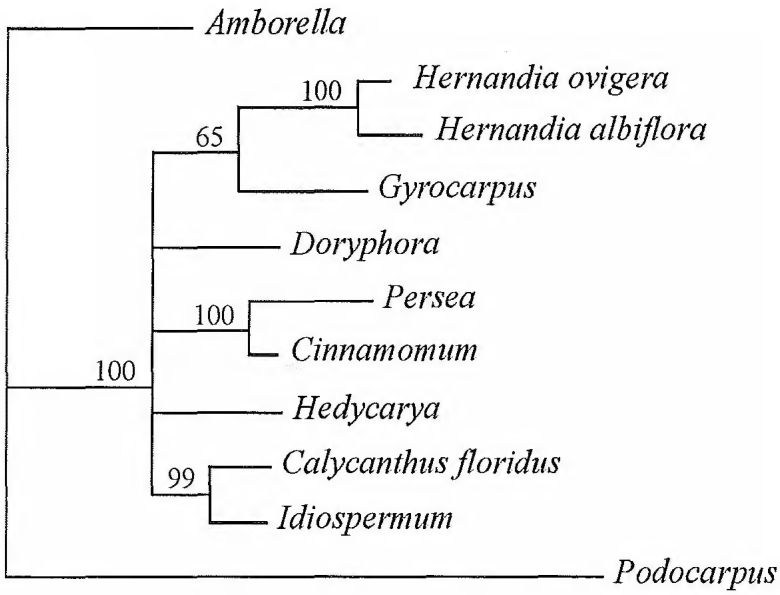

Fig. 3. Maximum likelihood analysis of *rbc*L sequences. The 50% majority tree of a 100 replicate bootstrap analysis is shown. Confidence estimates of groups are indicated by the numbers (percentage of bootstrap replicates with that particular group). See Table 1 for species information.

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